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Im Auftrag

For the President of the European Patent Office

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**In vivo assay and molecular markers for testing the phenotypic stability of cell populations, and
selected cell populations for autologous transplantation**

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**IN VIVO ASSAY AND MOLECULAR MARKERS FOR TESTING
THE PHENOTYPIC STABILITY OF CELL POPULATIONS, AND
SELECTING CELL POPULATIONS FOR AUTOLOGOUS
TRANSPLANTATION.**

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The present invention relates to the field of cartilage repair in general, and more specifically to the generation of an optimal cell population suitable for the repair of joint surface defects and the repair of the cartilage skeleton in general.

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BACKGROUND OF THE INVENTION

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Cartilage is a tissue composed by a cellular component, chondrocytes, and by an extra-cellular matrix typically rich in collagen type II and highly sulphated high molecular weight proteoglycan aggregates. The latter property confers cartilage its peculiar histochemical characteristics that are: strong staining with Alcian blue at low pH (from 0.2 to 2.5) and metachromacy with Toluidin blue and Safranin O. The abundance of type II collagen, link protein, and proteoglycan aggrecan, along with the presence of minor collagens such as type IX and type XI collagen are hallmarks of cartilage tissue.

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In post-natal mammals, cartilage contributes to the structure of several organs and systems like the articular surface of diarthrodial joints and other joint-associated structures (such as menisci), the ear, the nose, the larynx, the trachea, the bronchi, structures of the heart valves, part of the costae, synchondroses, entheses etc. In some of the mentioned locations (e.g. entheses, the annulus fibrosus of the intervertebral disks, in the menisci, insertion of ligaments etc.) for the abundance of collagens (mostly type I collagen) it is called *fibrocartilage*. In other locations (e.g. the pinna of the ear, epiglottis etc.) it is particularly rich of elastin and it is called *elastic cartilage*. In all the other structures, for its semi-transparent, clear aspect it is called *hyaline cartilage*.

In embryonic development mesenchymal cells aggregate and differentiate to form cartilage anlagen, which provide the mold of the future long bones.

These cartilage templates in development evolve, undergo endochondral bone formation through a cascade of events including chondrocyte hypertrophy, vascular invasion, mineralization, and are eventually replaced by bone except for a thin layer at the extremities of the bone elements that will differentiate into the articular surface of diarthrodial joints. In these locations cartilage tissue remains hyaline for all the life-span of the individual. With ageing, articular cartilage is well known to undergo a process of senescence, affecting its mechanical properties and its intrinsic resilience.

Joint surface defects can be the result of various aetiologies such as inflammatory processes, neoplasias, post-traumatic and degenerative events etc. Whatever the cause, the mechanisms of repair and of subsequent evolution are largely common.

Osteochondral (or full-thickness) articular surface defects include damage to the articular cartilage, the underlying subchondral bone tissue, and the calcified layer of cartilage located between the articular cartilage and the subchondral bone. They typically arise during severe trauma of the joint or during the late stages of degenerative joint diseases, e.g. during osteoarthritis. Since the subchondral bone tissue is both innervated and vascularised, damage to this tissue may be often painful. Osteochondral defects depend on the extrinsic mechanism for repair. Extrinsic healing relies on mesenchymal elements from subchondral bone to participate in the formation of new connective tissue. This repair tissue may undergo metaplastic changes to form fibrocartilage that does not however display the same biochemical composition or mechanical properties as normal articular cartilage or subchondral bone and degenerates with use.

Superficial or partial-thickness injuries of the articular cartilage that do not penetrate the subchondral bone rely on the intrinsic mechanism for repair. Soon after superficial injury, chondrocytes adjacent to the injured surfaces show a brief burst of mitotic activity associated with an increase in metabolic activity and matrix synthesis. Despite these attempts at repair, there is no appreciable increase in the bulk of cartilage matrix and the repair process is rarely effective in healing the defects. Although initially sometimes painful, partial-thickness defects often degenerate into osteoarthritis of the involved joint.

Repair of articular cartilage defects with suspensions of chondrocytes has been carried out in a variety of animal models (Brittberg et al. (1996)

Clin. Orthop. (326):270-83) and is now employed in humans (Brittberg et al. *N*

5 *Engl J Med.* 1994 Oct 6;331(14):889-95). Autologous chondrocytes obtained from an unaffected area of the joint are released, expanded *in vitro* in the presence of autologous serum and subsequently injected under a periosteal flap sutured to cover the cartilage defect. This procedure has led to a proven at least symptomatic amelioration. This promising approach has still wide margins for
10 improvement, since it is known that *in vitro* expansion of chondrocytes results, after a limited number of cell divisions, in a loss of their phenotypic stability (as defined by the ability of chondrocytes to form hyaline cartilage *in vivo*) making the cell suspension to be injected unreliable.

15 Three alternative approaches have been developed in an attempt to improve the success rate in treating mammalian articular cartilage defects. In the first approach, synthetic carrier matrices are impregnated with allogeneic chondrocytes and then implanted into the cartilage defect where they hopefully produce and secrete components of the extracellular matrix to form articular
20 cartilage at the site of the defect. A variety of synthetic carrier matrices have been used to date and include three-dimensional collagen gels (e.g. U.S. Pat. No. 4,846,835), reconstituted fibrin-thrombin gels (e.g. U.S. Pat. Nos. 4,642,120; 5,053,050 and 4,904,259), synthetic polymer matrices containing polyanhydride, polyorthoester, polyglycolic acid and copolymers thereof (U.S. Pat. No.
25 5,041,138), and hyaluronic acid-based polymers. Once a mitotically expanded population of chondrocytes is obtained, the cells can be implanted either back into the same subject from which their parent cells were originally derived (autologous implantation), or into a different subject (heterologous implantation). In addition, heterologous implantation may use chondrocytes obtained from a
30 related or unrelated individual of the same species (allogeneic), or from a different species (xenogeneic). Alternatively, chondrocytes may be obtained from an established, long-term cell line that is either allogeneic or xenogeneic.

The introduction of non-autologous materials into a patient, however, may stimulate an undesirable immun response directed against the implanted material, leading to potential rejection of the newly-formed and engrafted cartilage tissue. In addition, heterologous implantation risks the transmission to the subject of infectious agent(s) present in the tissue or cell line. Neo-cartilage may be formed around the periphery of the implant thereby preventing integration of the implant into the cartilage defect. Monitoring the formation and development of the resulting synthetic cartilage in situ is difficult to perform and usually involves an arthroscopic or open joint examination. Furthermore, implants containing synthetic polymer components may be unsuitable for repairing large cartilage defects since polymer hydrolysis in situ inhibits the formation of cartilage and/or its integration into the defect.

In the second approach, the defect is filled with a biocompatible, biodegradable matrix containing chemotactic and mitogenic growth factors to stimulate the influx of chondrocyte progenitor cells into the matrix in situ. The matrices optimally contain pores of sufficient dimensions to permit the influx into, and proliferation of the chondrocyte progenitor within the matrix. The matrix also may contain differentiating growth factors to stimulate the differentiation of chondrocyte progenitor cells into chondrocytes which in turn hopefully secrete extracellular matrix components to form cartilage at the site of the defect in situ (e.g. U.S. Pat. Nos. 5,206,023 and 5,270,300 and EP-A-530,804). This approach however results in problems similar to those associated with the first approach hereinabove. Furthermore there is no data so far that articular cartilage contains chondrocytic progenitors available for partial thickness defect repair.

In the third approach, chondrocytes may be cultured and expanded in vitro thereby to form synthetic cartilage-like material that is implanted subsequently into the cartilage defect. This has the advantage over the previous methods in that the development of the synthetic cartilage material may be monitored, through biochemical and morphological characterisation, prior to implantation. Growing chondrogenic cells may be achieved in either an anchorage-dependent or an anchorage-independent manner. In the latter, chondrogenic cells may be

cultured as colonies within an agarose gel. Heretofore, only small pieces of cartilage tissue of undefined shape have been prepared using this manner. Furthermore, the resulting cartilage remains embedded within a gel matrix making it less suitable for implantation into mammals. Alternatively, in another anchorage-independent method, chondrocytes may be cultured as colonies in suspension culture. However the resulting particles containing synthetic cartilage-like material are usually small and of undefined shape, and do not integrate with each other and with the surrounding cartilage within the defect. This makes them unsuitable for implantation and repair of a predetermined articular cartilage defect.

In the anchorage-dependent method, primary cultures of chondrogenic cells isolated from primary tissue are grown as monolayer attached to the surface of a cell culture flask (e.g. U.S. Pat. No. 4,356,261). The primary cells derived directly from explant tissue remain capable of producing and secreting extracellular components characteristic of natural cartilage, specifically type II collagen and sulphated proteoglycans. However, it is well known that during *in vitro* expansion as monolayers, chondrocytes dedifferentiate and lose their ability to form hyaline cartilage *in vivo*. Until now it has not been possible to prepare large patches of articular cartilage from small pieces of biopsy tissue using the anchorage-dependent procedures of U.S. Pat. No. 4,356,261.

In order to solve the above problems, U.S. Pat. No. 5,723,331 provides a method for preparing *in vitro* large quantities of synthetic cartilage from small samples of biopsy tissue which, based on the discovery that chondrogenic cells may be isolated from a variety of tissues, e.g. pre-existing cartilage, perichondrial tissue or bone marrow, and expanded *in vitro* prior to cartilage formation, includes first seeding denuded (i.e. isolated from an enzymatically or mechanically disaggregated tissue) chondrogenic cells, proliferated *ex vivo*, into a pre-shaped well having a cell contacting, cell adhesive surface, and then culturing the proliferated chondrogenic cells in the well for a time sufficient to permit the cells to secrete an extracellular matrix thereby to form a three-dimensional, multi cell-layered patch of synthetic cartilage. This approach does

not yield an optimal integration between the implant and the surrounding cartilage. This far there is no evidence on the phenotypic stability of cells in such preparations.

5 The use of mesenchymal cells has also been proposed for cartilage repair.

 Mesenchymal cells are a potential alternative source of cartilage-producing cells. They are generally recognised as pluripotent cells capable of dividing many times to produce progeny cells that can eventually give rise to
10 many tissues, including skeletal tissues such as cartilage, bone, tendon, ligament, marrow stroma and connective tissue. By definition, they are not limited to a fixed number of mitotic divisions. However stem cells are defined as cells which are not terminally differentiated, which can divide without limit to yield
15 cells that are either stem cells or which irreversibly differentiate to yield a new type of cell. Those stem cells which give rise to a single type of cell are called unipotent cells; those which give rise to many cell types are called pluripotent cells. Chondro/osteoprogenitor cells, which are bipotent with the ability to differentiate into cartilage or bone, were isolated from bone marrow (e.g. in U.S. Pat. No. 5,226,914). Pluripotent mesenchymal stem cells were subsequently
20 isolated from muscle, heart and granulation tissue. Pluripotency is demonstrated using a non-specific inducer, dexamethasone, which elicits differentiation of the stem cells into chondrocytes (cartilage), osteoblasts (bone), myotubes (muscle), adipocytes (fat), and connective tissue cells.

25 Unfortunately, although it is highly desirable to have stem cells which are easily obtained by a muscle biopsy, cultured to yield large numbers, and can be used as a source of chondrocytes or osteoblasts or myocytes, there is no known specific inducer of the mesenchymal stem cells that yields only cartilage. In vitro studies in which differentiation is achieved yields a mixture of cell types. In U.S.
30 Pat. Nos. 5,226,914 and 5,197,985 the cells were absorbed into porous ceramic blocks and implanted, yielded primarily bone. However, U.S. Pat. No. 5,906,934 discloses that under very specific conditions mesenchymal stem cells in a suitable polymeric carrier (such as polyglycolic acid mesh) implanted into a

cartilage and/or bone defect will differentiate to form cartilage and/or bone, as appropriate. Also U.S. Pat. No. 5,919,702 discloses chondrocyte progenitor cells isolated from umbilical cord sources, e.g. from Wharton's jelly, and cultured so as to give rise to chondrocytes that can produce cartilage tissue. Also in another attempt to avoid the drawbacks of current cartilage and bone repair techniques which cause bleeding and involve the use of mechanically weak non self-derived material, U.S. Pat. No. 5,866,415 suggests treating cartilage or bone defects with a biological material obtained by attaching in vitro cartilage or bone forming cells to a periosteum of sufficient size to accommodate the defect.

Transforming growth factor- β ("TGF- β ") refers to a family of related dimeric proteins which regulate the growth and differentiation of many cell types. Members of this family include TGF- β 1, TGF- β 2, TGF- β 3, TGF- β 4, TGF- β 5, morphogenic proteins ("MP") such as MP-121 and MP-52, inhibins/activins (such as disclosed in EP-A-222,491), osteogenic proteins ("OP"), bone morphogenetic proteins (hereinafter denoted "BMP"), growth/differentiation factors ("GDF") such as GDF-1, GDF-3, GDF-9 and Nodal. TGF- β was first characterised for its effects on cell proliferation. It both stimulated the anchorage-independent growth of rat kidney fibroblasts and inhibited the growth of monkey kidney cells. TGF- β family members have been shown to have many diverse biological effects, e.g. they regulate bone formation, induce rat muscle cells to produce cartilage-specific macromolecules, inhibit the growth of early haematopoietic progenitor cells, T cells, B cells, mouse keratinocytes, and several human cancer cell lines. TGF- β family members increase the synthesis and secretion of collagen and fibronectin, accelerate healing of incisional wounds, suppress casein synthesis in mouse mammary explants, inhibit DNA synthesis in rat liver epithelial cells, stimulate the production of BFGF binding proteoglycans, modulate phosphorylation of the epidermal growth factor ("EGF") receptor and proliferation of epidermoid carcinoma cells and can lead to apoptosis in uterine epithelial cells, cultured hepatocytes and regressing liver. TGF- β s can mediate cardio-protection against reperfusion injury by inhibiting neutrophil adherence to endothelium and protect against experimental autoimmune diseases in mice. On the whole, proteins of

the TGF- β family are multifunctional, hormonally active growth factors and also have related biological activities such as chemotactic attraction of cells, promotion of cell differentiation and tissue-inducing capabilities. Differences in their structure and in their affinity for receptors lead to considerable variations in their exact biological function.

In contrast to the foregoing reports of the ability of TGF- β to induce the production of cartilage-specific macromolecules in muscle cells and chondrocytes, TGF- β was found to act synergistically with fibroblast growth factor to inhibit the synthesis of collagen type II by chicken sternal chondrocytes and in rat chondrocytes. In fact, TGF- β has emerged as the prototypical inhibitor of the proliferation of most normal cell types in vitro as well as in vivo, exhibiting a remarkable diversity of biological activity. TGF- β 1 has been purified from human and porcine blood platelets and recombinant TGF- β 1 is currently available.

Among the sub-family of BMPs, the structures of BMP-1 through BMP-13 have previously been elucidated. The unique inductive activities of these proteins, along with their presence in bone, suggests that they are important regulators of bone repair processes and may be involved in the normal maintenance of bone tissue. Recently, the BMP-12-related subfamily of proteins, including BMP-13 and MP52 (see e.g. WO93/16099 and U.S. Pat. No. 5,658,882), was shown to be useful in compositions for the induction of tendon/ligament-like tissue formation and repair. U.S. Pat. No. 5,902,785 discloses that BMP-12 related proteins are particularly effective for the induction of cartilaginous tissue and that BMP-9 is useful for increasing proteoglycan matrix synthesis and therefore for the maintenance of cartilaginous tissue. It also describes compositions comprising a BMP-12 related protein and additionally including one or more TGF- β proteins proven to be osteogenic, preferably BMP-2, -4, -5, -6 and/or BMP-7 as useful for the regeneration of multiple tissue types (for example at the interface or junction between tissues) and especially useful for the treatment of articular cartilage, in which the articular surface, cartilage, subchondral bone and/or tidemark interface between cartilage and bone need to

be repaired. The same patent further describes compositions comprising a BMP-12 related protein together with a protein useful for the maintenance of chondrocytes or cartilaginous tissue such as BMP-9, the said compositions being especially useful for the induction and maintenance of cartilaginous tissue at a site in need of cartilage repair such as an articular cartilage defect.

WO96/14335 discloses, using mRNA prepared from newborn articular cartilage, the isolation of two members of the BMP family, designated Cartilage-derived morphogenetic proteins-1 and -2 (CDMP-1, CDMP-2). Storm et al. (1994) in *Nature* 368, 639-43 and Chang et al. (1994) in *J.Biol.Chem.* 269, 28227-34 independently established that CDMP-1 mapped close to the brachypodism locus on chromosome 2 in mice and might be involved in the brachypodism phenotype. Also the expression patterns of CDMP's suggests an important role for these genes in joint morphogenesis. WO98/59035 also discloses a method of maintaining a cartilaginous phenotype in chondrocytes *in vitro*, comprising culturing the chondrocytes in serum-free medium containing a CDMP and/or BMP.

A table summarising the TGF β superfamily members follows (Reddi AH, *Nature Biotechnol.* 1998, 16:247-52).

The BMP family in mammals

BMP subfamily	Generic name	BMP designation
BMP 2/4	BMP-2A	BMP-2
	BMP-2B	BMP-4
BMP 3	Osteogenin	BMP-3
	Growth/differentiation factor 10	BMP-3B
Op- 1/BMP-7	BMP-5	BMP-5
	Vegetal related-1 (Vgr-1)	BMP-6
	Osteogenic Protein-1 (Op-1)	BMP-7
	Osteogenic Protein-2 (Op-2)	BMP-8
	Osteogenic Protein-3 (Op-3)	BMP-8B
	Growth/differentiation factor 2 (GDF- 2)	BMP-9
	BMP-10	BMP-10
	Growth/differentiation factor 11 (GDF- 11)	BMP-11
	Growth/differentiation factor 7 (GDF- 7) or cartilage-derived morphogenetic protein-3 (CDMP-3)	BMP-12
	Growth/differentiation factor 6 (GDF- 6) or cartilage-derived morphogenetic protein-2 (CDMP-2)	BMP-13
GDF- 5,6,7	Growth/differentiation factor 5 (GDF- 5) or cartilage-derived morphogenetic protein-1 (CDMP-1)	BMP-14
	BMP-15	BMP-15

Other families of growth factors have been shown to play a role in cartilage formation/differentiation. Among them the fibroblast growth factors (FGFs) are a family of polypeptide growth factors involved in a variety of activities. One of their receptors, FGF receptor 3 (FGFR3) (Keegan K. et al., 1991 Proc. Nat. Acad. Sci. 88: 1095-99), is known to play a crucial role in chondrogenesis. Point mutations in the *fgfr3* gene resulting in a ligand-independent constitutively active protein (which means that the FGF signalling is always active also in the absence of the ligand) cause skeletal abnormalities as achondroplasia and thanatophoric dysplasia.

As already outlined in page 3, although autologous chondrocyte transplantation ("ACT") is becoming a widely accepted technique for repair of joint surface defects ("JSD") it still presents some drawbacks. More in details, this procedure implies *in vitro* expansion - in the presence of autologous serum - of autologous chondrocytes obtained from an uninvolved area of the joint surface, followed by the implantation of the chondrocyte suspension under a periosteal flap sutured to seal the joint surface defect. Cell expansion is necessary to obtain from a small cartilage biopsy a number of cells sufficient to repair the cartilage defect. To date, however, it is not known how far it is possible to expand chondrocytes without hampering their phenotypic stability, i.e. their capability of organising hyaline cartilage *in vivo*. Indeed it is well known, as explained before, that *in vitro* expansion of chondrocytes results in cell de-differentiation. This implies that chondrocyte expansion pays the price of loss of phenotypic stability and therefore the need of a quality control on expanded chondrocytes to be used for ACT. At the end of cell expansion the chondrocyte population is composed of some cells that retain their phenotypic stability, and others that still can proliferate but will not anymore contribute to cartilage repair. In order to obtain a consistent cell suspension for ACT, it is desirable to select stable chondrocytes within the expanded cell population. Chondrocytes are skeletal cells able to grow in anchorage-independent agarose cultures. The ability of chondrocytes to grow in anchorage-independent conditions is critical for those cells to survive and organise cartilage tissue once injected as a cell suspension for repair of JSD, but is probably not the only necessary phenotypic

trait.

SUMMARY OF THE INVENTION

The issues explained above clearly show that there is a need for an
5 *in vivo* assay to measure the phenotypic stability of chondrocytes and at the
same time their capacity to grow in anchorage-independent conditions. Among
skeletal cells the anchorage independent growth is peculiar of chondrocytes and
chondrocytic precursors. Therefore this property is required only when
chondrocyte-like cells are of interest. There is also a need to identify molecular
10 markers associated with specific cell types that would allow the clinician to
regenerate and repair cartilage tissue with the appropriate cells and avoid scar
formation to the greatest possible extent. These goals and other purposes are
achieved by means of the following objects of the present invention.

15 A first object of the present invention is to provide an *in vivo* assay
to measure anchorage independent growth and phenotypic stability of a certain
cell population, and more specifically to measure at the same time the
anchorage-independent growth of cells and their potential to retain their
commitment to a certain (original or induced by manipulation) differentiation
20 pathway. A second object of the invention is the use of the aforesaid *in vivo*
assay to evaluate the risk that a certain procedure or treatment administered to a
certain cell population can hamper its anchorage-independent growth as well as
its phenotypic stability. A third object of the invention is the use of the aforesaid
in vivo assay to predict the outcome of autologous chondrocyte transplantation
25 ("ACT") using a certain population of expanded chondrocytes. A fourth object of
the invention is the use of the aforesaid *in vivo* assay to identify molecular
markers linked to the phenotypic stability of a certain cell population. A fifth
object of the invention is the definition of a set of molecular markers linked to the
outcome of the aforesaid *in vivo* assay using freshly isolated chondrocytes and
30 therefore linked to the chondrocyte stability. A sixth object of the invention is the
use of these positive and negative markers of chondrocyte stability as a tool to
monitor, passage by passage, *in vitro* cell expansion. This tool will be useful to
predict when cell expansion must be stopped, to recover chondrocytes that have

already lost their phenotypic stability only when needed, and especially to provide a quality control for chondrocytes to be used for ACT, and therefore will make chondrocyte suspensions for ACT a more reliable and consistent product. A seventh object of this invention is the use of FACS (Fluorescence Activated Cell Sorting) analysis and cell sorting using positive and negative markers to select, from a chondrocyte population, only those cells that retain their phenotypic stability. Another object of this invention is the use of cells selected from a cell population as mentioned above for a variety of clinical applications including transplantation into a patient through surgery or arthroscopic injection, namely to promote the repair or regeneration of damaged joints or joint surfaces, or seeding prosthetic devices. Yet another object of this invention is a therapeutic composition including cells selected by the above method for use in the said clinical applications.

15 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1. is a set of 4 pictures showing the histological and histochemical characteristics of implants from the *in vivo* assay of the invention.

Figure 2. is a picture showing *in situ* hybridisation for human-specific Alu repeats of an implant from the *in vivo* assay of the invention.

20 Figure 3. is a picture showing that epiphyseal chondrocytes injected in the *in vivo* assay yield hypertrophic cartilage with foci of endochondral bone formation.

Figure 4. is a picture showing an agarose culture of late passage articular chondrocytes.

25 Figure 5. is a picture showing the results of RT-PCR analysis for various molecular markers in freshly isolated and passaged chondrocytes.

Figure 6. is a set of 2 pictures showing implants obtained by injecting chondrocytes previously treated with or without CDMP1.

30 DETAILED DESCRIPTION OF THE INVENTION

Terms used throughout this disclosure are defined as follows:

Chondrocyte stability

The capacity of a cell suspension (either obtained from cartilage tissue or

from any other tissue containing cells with chondrogenic potential) to produce a hyaline cartilage implant without signs of vascular invasion or endochondral bone formation 3 weeks after its intra-muscular injection in immune-deficient mice.

Chondrogenic

- 5 The capacity to promote or stimulate cartilage growth, as applied to cells such as chondrocytes and to cells which themselves differentiate into chondrocytes.

Connective tissue

- 10 Any structural tissue in the body of a mammal including bone, cartilage, ligament, tendon, meniscus and joint capsule.

Differentiation

- 15 A biological process by which primitive unspecialised cells undergo a series of cellular divisions giving rise to progeny having more specialised function(s). Terminal differentiation provides a highly specialised cell having unique functional and phenotypic characteristics.

Phenotypic stability

- 20 The capacity of any cell to reorganise *in vivo* the structure of a specific tissue, either the original tissue where the cells were taken from, or a different tissue the cells have been forced to form under specific conditions.

- 25 The present invention is based upon unexpected discoveries. First, chondrocyte stability can be measured *in vivo* and linked to a set of molecular markers. Secondly this set of molecular markers is associated to the outcome of joint surface defects ("JSD") repair in well-standardised animal models of JSD. Thirdly, membrane-associated molecular markers can be used to select, from an expanded chondrocyte population, only those cells that, retaining their phenotypic stability, will be able to optimally repair JSD. The set of molecular markers (both membrane-associated and non membrane-associated) will also be used as a final quality control for the cell suspension to be used for ACT or
- 30 the repair of the cartilaginous structures, thus providing a reliable and consistent final product.

A first embodiment of the present invention consists of an *in vivo* assay

which measures the capacity of isolated cells to reproduce *in vivo* the architecture of a tissue with all its cellular and extra-cellular components, i. . all its specific characteristics. This assay – originally devised to measure chondrocyte stability but extendable to any cell population involved in a certain differentiation pathway - consists of an *in vivo* assay to measure anchorage-independent growth and phenotypic stability of a certain cell population comprising subcutaneous or intramuscular injection in a mammal of a cell suspension of articular chondrocytes in an iso-osmotic liquid, the same suspension comprising articular chondrocytes in an amount equivalent to at least 1×10^6 (preferably from 2×10^6 to 20×10^6) chondrocytes as applied to immune-deficient mice. In the case that the mammal is a mouse, the *in vivo* assay consists of the injection of a single cell suspension intramuscularly in immune-deficient nude mice. After a certain period of time, at least 3 weeks, the mouse is sacrificed, dissected, and the implant, if retrieved, weighed, fixed and histologically evaluated. The *in vivo* assay of the invention is highly specific since about 5×10^6 freshly isolated articular chondrocytes injected in a volume of 50-100 μ l of any iso-osmotic liquid such as phosphate buffered saline (PBS) or HBSS, are sufficient to yield after 3 weeks an implant of mature cartilage. The same number of expanded periosteal cells yield a fibrous tissue histologically resembling periosteal tissue. On the contrary, the injection of cell lines known to have *in vitro* osteo-chondrogenic potential – namely ATDC5, CFK2, RCJ, and C5.18 cells – did not yield any retrievable implant. Importantly, serially passaged (P4 and P5) articular chondrocytes, still retaining their anchorage-independent growth as measured by agarose culture (according to the method of Benya et al. (1982) in *Cell* (1):215-24) failed to yield any implant. Strikingly epiphyseal chondrocytes (which in normal embryonic development undergo endochondral ossification and are destined to be substituted by bone) yield a cartilaginous implant in which vascular invasion, chondrocyte hypertrophy and bone formation are taking place.

A second embodiment of the invention is the use of certain specific conditions of the *in vivo* assay of the first embodiment to evaluate the possibility that a certain procedure or treatment administered to a certain cell population

involved in a certain differentiation pathway can hamper its anchorage-independent growth as well as its phenotypic stability. For instance, while enzymatic digestion induces no such risk, on the contrary storage in liquid nitrogen or extensive cell expansion (after 2-3 passages) compromises the ability of chondrocytes to yield a cartilaginous implant in the *in vivo* assay. On the other hand, the *in vivo* assay also evaluates whether a certain treatment, such as addition of growth factors/reagents, or procedure, such as physical stimulation, enhances the phenotypic stability of cell populations. For instance, treating the cell suspension for 30 minutes with CDMP-1 (100 ng/ml) just before injection, followed by washing two times in PBS, resulted in a three fold increase in the wet weight of the retrieved implant as compared to control injections, and in a 2 fold increase in the number of cells. Such enhancement can allow a dramatic reduction of the expansion needed for JSD repair (in some cases and ultimately making *in vitro* expansion not needed at all) and consequently a corresponding reduction of the risk to make chondrocytes phenotypically unstable.

A third embodiment of the invention is the use of the *in vivo* assay of the first embodiment to predict the outcome of autologous cell transplantation ("ACT") using a certain population of cells involved in a certain differentiation pathway (e.g. expanded chondrocytes) as a means to predict phenotypic stability (e.g. chondrocyte stability). This can be evaluated either using well-standardised animal models for ACT or using an *ex vivo* system. This *ex vivo* system consists of placing articular cartilage, with or without underlying bone, in culture (liquid, solid or semi-solid), producing a cartilage defect, with or without a natural or synthetic membrane to cover the lesion, and applying, underneath the membrane, a cell population either in suspension, or seeded within a carrier, with or without growth factors to mimic *in vitro* the events that take place *in vivo* during JSD repair.

A fourth embodiment of the invention is the use of the *in vivo* assay of the first embodiment to identify molecular markers linked to the phenotypic stability of a certain cell population involved in a certain differentiation pathway, e.g. chondrocytes. Those molecular markers can be identified by semi-quantitative

RT-PCR, by Northern hybridisation (as explained in example 4 below), by the generation of subtracted libraries from cell population that succeed in the *in vivo* assay matched to similar cell populations that fail (e.g. serially passaged chondrocytes), by differential display or subtractive hybridisation approaches, or
5 by DNA arrays or DNA chips.

A fifth embodiment of the invention is the identification of a set of molecular markers linked to the outcome of the *in vivo* assay of the first embodiment, using freshly isolated or serially passaged cells from a certain cell
10 population involved in a certain differentiation pathway, e.g. chondrocytes, and therefore linked to the phenotype (e.g. chondrocyte) stability. For instance, freshly isolated human chondrocytes were used for RNA purification and cultivated *in vitro*. Upon passaging, an aliquot of cells was used for RNA purification, 2 aliquots of 5×10^6 cells were injected in the *in vivo* assay and the
15 rest re-plated. RNAs were tested by semi-quantitative RT-PCR for expression of genes known to have a role in chondrogenesis and cartilage maintenance.

In the PCR analysis were also included genes isolated from a subtracted cDNA population obtained by a subtractive hybridization approach: cDNA from pig P0 chondrocytes (stable in the *in vivo* assay) was matched against cDNA
20 from P1 chondrocytes (that failed to yield an implant) in a two-way subtractive hybridisation. Individual cDNAs from both subtracted cDNA populations (P0-P1 and P1-P0) were cloned in PCR-Script Amp SK(+) vector, and sequenced. The human homologs, when known, were included in the RT-PCR analysis. Unknown cDNAs were evaluated for differential expression by Northern analysis.

25 Results indicate the high expression of BMP-2, FGFR3, and type II collagen as positively associated to chondrocyte stability, whereas activin-like kinase (ALK)-1 and collagen type X expression are negatively associated. Interestingly CDMP-1 expression appears upon plating reaching a plateau to disappear again late after passage 4.

30

A sixth embodiment of the invention is the use of the positive and negative markers of phenotype (e.g. chondrocyte) stability identified in the fifth embodiment as tools to monitor passage by passage cell expansion, namely to

predict when cell expansion must be stopped and/or to recover cells (e.g. chondrocytes) that have already lost their phenotypic stability only when needed, and eventually to provide a means for quality control of cells (e.g. chondrocytes) to be used for autologous cell transplantation ("ACT"), thus making cell (e.g. chondrocyte) suspensions for ACT a more reliable and consistent product.

A seventh embodiment of this invention is the use of FACS analysis and cell sorting to select, from a cell (e.g. chondrocyte) population, only those cells that retain their phenotypic stability. "Positive" membrane-associated markers (e.g. FGFR-3) will be used for positive selection of cells with phenotypic stability (e.g. stable chondrocytes), while "negative" membrane-associated markers (e.g. ALK-1) will be used to sort out cells without phenotypic stability (e.g. unstable chondrocytes). The consistency of the selection will be monitored by the detection of unrelated, non membrane-associated markers such as BMP-2 and type II collagen in the sorted population, thus significantly enriching the cell population to be used for ACT with cells with phenotypic stability (e.g. stable chondrocytes) and consequently increasing quality and efficiency of the whole procedure.

Another embodiment of this invention consists in using cells retaining phenotypic stability and selected from a cell population by means of the above selection method for a variety of clinical applications. For instance, they may be transplanted without further processing to a connective tissue site in a patient to promote the repair or regeneration of damaged bone or cartilage. Unlike previous methods, the present invention does not necessarily require (as explained in the second embodiment) *in vitro* culturing in order to obtain a suitable (both in nature and quantity) cell population for use for *in vivo* application. By way of example, the said selected cells retaining phenotypic stability may be implanted at any connective tissue site needing cartilage regeneration by any implanting procedure such as surgery or arthroscopic injection. Another clinical application of such cells involves seeding any prosthetic device intended to be anchored into a mammal host in order to improve the attachment of the said device. This includes knee and hip

replacement devices made from organic or inorganic materials having low immunogenic activity such as titanium alloys, ceramic hydroxyapatite, stainless steel and cobalt-chrome alloys.

5 Yet another embodiment of this invention consists of a therapeutic composition including cells selected by the above method for use in the said clinical applications. In addition to the selected cells, the composition usually includes at least a pharmaceutically acceptable carrier, well known to those skilled in the art and for instance selected from proteins such as collagen or
10 gelatine, carbohydrates such as starch, polysaccharides, sugars (dextrose, glucose and sucrose), cellulose derivatives like sodium or calcium carboxymethylcellulose, hydroxypropyl cellulose or hydroxypropylmethyl cellulose, pregeletanized starches, pectin agar, carrageenan, clays, hydrophilic gums (acacia gum, guar gum, arabic gum and xanthan gum), alginic acid,
15 alginates, hyaluronic acid, dextran, pectins, synthetic polymers such as water-soluble acrylic polymer or polyvinylpyrrolidone, proteoglycans, calcium phosphate and the like. When the therapeutical composition is intended for transplantation to a site in the body needing repair, it may additionally include at least one growth factor of the TGF- β family.

20

A more complete understanding of the present invention will be obtained by referring to the following illustrative examples.

Example 1 - cartilage obtainment and cell isolation

Articular cartilage was obtained, within 24 hours after death unless
25 otherwise indicated from human donors not having suffered from any articular disease. After macroscopic inspection to rule out gross joint pathologies, cartilage was sliced full thickness from femoral condyles and placed in Hank's Balanced Salt Solution ("HBSS") (available from Life Technologies) supplemented with 200 units/ml penicillin, 200 μ g/ml of streptomycin, and 0.5
30 μ g/ml of amphotericin B (Life Technologies). After two washes in HBSS during 5 minutes at 37°C, cartilage was finely minced and placed in a sterile 0.2% crude collagenase (Life Technologies) solution in Dulbecco's Modified Eagle Medium ("DMEM") with high glucose (Life Technologies) containing 10% foetal bovine

serum ("FBS") (Biowittaker), 200 units/ml penicillin, 200 µg/ml of streptomycin, and 0.5 µg/ml of amphotericin B. After overnight incubation at 37°C, cells were washed twice in culture medium - DMEM supplemented with 10% FBS, 100 units/ml penicillin, 100 µg/ml of streptomycin, and 0.25 µg/ml of amphotericin B - and counted with trypan-blue exclusion test to adjust for the number of viable cells.

Example 2 - *In vivo* assay

Cells isolated in example 1 were washed twice in sterile phosphate buffered saline ("PBS"), resuspended in a volume of 100 µl of PBS and injected intramuscularly in the thigh of female, 4-5 weeks old immune-deficient nude mice. Animals were sacrificed after 3 weeks by cervical dislocation and the thigh dissected to retrieve the presence of the implant in the site of injection. Implants were weighed, and either snap-frozen and stored in liquid nitrogen for *in situ* hybridisation or fixed in freshly-made 4% formaldehyde for 4 hours for histology and immunohistochemistry. After fixation the samples were included in paraffin, cut in 5 µm thick sections and coloured according to standard protocols (Alcian blue pH 2.5, Toluidin blue, Masson's trichrome, Safranin O) (Manual of Histological Techniques). Different amounts of cells, from 20×10^6 to 5×10^5 were used for injection in order to establish the minimum amount of cells that yielded a cartilage implant. Although the minimal amount of freshly isolated chondrocytes that yielded an implant was 1×10^6 , as an optimal amount we chose to use 5×10^6 cells because this number always yielded at least one implant in duplicate injections when freshly isolated chondrocytes were used. Although the wet weight of the implant tended to be higher when more cells were injected, it was difficult to draw a complete dose response curve since cell suspensions with more than 10×10^6 cells were very dense and difficult to inject reliably.

Figure 1 shows that implants appeared made of well differentiated hyaline cartilage rich in highly sulphated proteoglycans - as demonstrated by the Alcian blue staining at pH 2.5 (fig. 1A), strong metachromatic staining with Toluidin blue (Fig. 1B) and Safranin O (Fig 1C) - and devoid of any sign of bone formation as demonstrated with Masson's trichrome staining or vascular invasion (Fig. 1D). In order to check that viable cells were needed to organise the cartilage implant, we injected an equal number of cells that had been killed by freezing and thawing

three times in liquid nitrogen. Those injections yielded no implant. We also investigated whether cells should be able to proliferate, we irradiated freshly isolated chondrocytes with a single dose of 50 Gy, a dose that blocks proliferation but is not lethal to the cells. Except for some cytological atypies, the
5 injections yielded an otherwise normal hyaline cartilage implant.

In order to investigate whether the cartilage implant is made of cells of human origin, i.e. to rule out that the only role of the injected cells is producing factors that induce chondrogenesis in the mouse muscle), we performed *in situ* hybridisation for human specific Alu repeats as described by Kuznetsov et
10 al.(1997), *J Bone Miner. Res.*(9):1335-47. This procedure demonstrated that cells contributing to cartilage formation in our *in vivo* assay are of human origin, i.e. derive from the injected cells and not from the mouse host. As shown in figure 2 the nuclei of the cartilaginous implant (lower right part of the picture) stain positive – black – for the presence of human specific Alu repeats while the
15 muscle, pertaining to the mouse host, is completely negative.

Strikingly, as shown in figure 3, the injection of embryonic epiphyseal chondrocytes (which in normal embryonic development are replaced by bone) yield implant with vascular invasion and endochondral bone formation. These data demonstrate the fine specificity of the *in vivo* assay in reporting the
20 phenotypic pathway the injected cell is placed in.

Example 3: serial passaging results in impaired chondrocyte stability.

Cartilage samples from 3 independent human donors and 2 pig donors were placed in monolayer culture. Upon passaging, an aliquot of cells was destined to duplicate injection in the *in vivo* assay of example 2 and to RNA
25 isolation. Chondrocyte stability, as measured by the retrieval of a cartilage implant after 3 weeks in the site of injection, was lost between passage 1 and 3. In order to investigate whether anchorage-independent growth was impaired, we established agarose cultures with two independent samples at P4 that had already lost their phenotypic stability at P1 and P2. Another aliquot of these cells
30 was injected in the *in vivo* assay of example 2 and RNA was also isolated. No implant was retrieved from mice injected with these cells. As shown in figure 4, colony formation became evident after 3 weeks in agarose *in vitro* cultures demonstrating that anchorage-indep ndent growth was still possible far beyond

the loss of phenotypic stability.

Example 4: molecular markers associated with chondrocytic stability

Three pools of human articular chondrocytes were obtained as described in example 1 and cultured in monolayer. Upon passaging, 2 aliquots (5×10^6 cells each) were injected in the *in vivo* assay of example 2, a smaller aliquot was used to obtain the RNA extract and the rest was re-plated. Total RNAs were reverse-transcribed using Thermoscript (available from Life Technologies) and used for semi-quantitative PCR analysis. After passage 4, two samples were placed in low melting-agarose cultures, a system known to result in a rescue of type II collagen expression by de-differentiated chondrocytes. After 2 months, colony formation was abundant and cultures were harvested for RNA extraction. Semi-quantitative RT-PCR analysis was carried out for expression of genes involved in chondrogenesis.

In order to explore the role of genes unknown to be involved in chondrogenesis, we also undertook a differential expression analysis based on the principle of subtractive hybridisation: pig articular chondrocytes were plated and cultured in monolayer. Upon passaging cells were assayed for chondrocyte stability and RNA was isolated. Poly A⁺ RNA was purified using Oligotex mRNA Mini Kit (available from Quiagen) from total RNA derived from P0 and P1 cells. We chose those two populations because P0 cells still yielded a cartilage implant in the *in vivo* assay of example 2 while P1 cells did not. cDNAs were reciprocally subtracted in order to obtain species differentially expressed by P0 cells (potential positive markers of stable chondrocytes) and species differentially expressed by P1 cells (negative markers). Subtraction and amplification of subtracted cDNAs were performed using PCR-SelectTM cDNA subtraction Kit (available from Clontech). cDNAs were cloned in PCR Script amp SK (+) vector and sequenced. Genes of which human homologue was known were included in the semi-quantitative RT-PCR analysis on human samples, while unknown genes were controlled for their differential expression in the original RNA population by Northern analysis. The detailed procedures used were as follows:

RNA preparation

Total RNA from chondrocytes was isolated using Trizol reagent (available from Life Technologies), ethanol precipitated and stored at -70°C for further use.

Total RNA from agarose cultures was obtained by homogenising the whole culture in 6M urea, 3M lithium chloride with a Polytron homogeniser, and the major part of agarose was removed by centrifugation at room temperature at 3000 rpm for 15 minutes. Nucleic acids in the supernatant were allowed to
5 precipitate overnight at 4°C, pelleted by centrifugation 15 minutes at 18000 rpm at 4°C, supernatant was removed, RNA was air dried and dissolved in RNase-free water. Residues of agarose and other contaminants were removed by phenol-chlorophorm extraction followed by ethanol precipitation. Samples were re-dissolved in RNase-free water and stored at -70°C for further use. For those
10 samples requiring mRNA selection, poly A⁺ tailed RNA was sorted out of total RNA by double selection using Oligotex mRNA Mini Kit (Quiagen).

Semi-quantitative RT-PCR analysis

1 µg of total RNA was first strand-transcribed using Thermoscript (Life technologies). Before PCR analysis, cDNAs were equalised for β actin. PCR for
15 human β actin was carried out in a volume of 10 µl stopping the reaction after 18, 19, 20 cycles to make sure that PCR amplification was still in an exponential phase. PCR products were electrophoresed in 1% agarose gel in TBE buffer, stained with ethidium bromide and the intensity of the bands was analysed by densitometry using Image Master software (available from Pharmacia-Biotech).
20 cDNAs were diluted according to the relative intensity of the bands. To rule out that β actin was differentially regulated in the different samples to be compared, the same analysis was also performed for GAPDH mRNA. After equalisation for β actin, all samples were simultaneously tested for a number of genes known to be involved in chondrogenesis and cartilage maintenance. The same analysis
25 was performed for those molecules obtained with a subtractive hybridisation approach. For each gene, cycling was optimised in such a way that amplification was still in an exponential phase when PCR was stopped for all samples.

Freshly isolated articular chondrocytes were cultured in monolayer and serially passaged. Upon passaging, chondrocytes were tested in the *in vivo*
30 assay and RNA was isolated, first strand transcribed and used for PCR analysis. As showed in figure 5, the expression of FGFR-3 and BMP-2 dramatically decreases after loss of phenotypic stability as measured by the *in vivo* assay, correlating tightly with collagen type II expression (a molecule known to be

expressed at high levels by fully differentiated chondrocytes). The expression of activin-like kinase-1 (ALK-1) and collagen type X, on the contrary, progressively increases in time. Cdmp1 reaches the plateau after plating and disappears only after passage 4. The vertical white bar in figure 5 marks the last passage when cells retained their phenotypic stability therefore bands to the left correspond to stable chondrocytes (early passages), while bands on the right of the white bar correspond to late passage chondrocytes that have lost phenotypic stability. Interestingly, expression of BMP-2 and FGFR-3, as well as collagen type II was completely rescued in late passage chondrocytes when cultivated for 1 month in low temperature-melting agarose anchorage-free cultures as described above.

Northern Analysis

Denaturing gel electrophoresis of RNA was performed as follows: 5 µg of total RNA from each sample were dissolved in 0.5M MOPS, 0.01M NaEDTA (pH 7.0) containing 2.2M formaldehyde and 50% by volume formamide, denatured at 70°C for 10 minutes, chilled on ice and immediately loaded onto a 1.5% agarose gel in MOPS/EDTA, 6.6% formaldehyde. Samples were electrophoresed during 3 hours at 10 V/cm, blotted on HybondTM N membranes (Amersham Pharmacia Biotech) and UV cross-linked. [³²P] dCTP-labelled probes were generated from eluted PCR products of the individual clones using a Prime-it kit commercially available from Stratagene. Blotted membranes were hybridised in Quikhyb hybridisation buffer (Stratagene) at 60°C for 1 hour and washed to a final stringency of 65°C in 0.1x SSC 0.5x SDS. Autoradiographic films BiomaxTM MS (Kodak) were exposed at -70°C for 24 hours and developed.

Example 5 - the *in vivo* assay and the set of markers for predicting the outcome of autologous chondrocyte transplantation (ACT) in an animal model.

Male young New Zealand white rabbits or goats are used as a model of ACT. Articular cartilage of the patella or femoral condyles are carved with a device producing a superficial cartilage defect 0.3 mm deep and 3 mm large in diameter, therefore not penetrating the underlying bone. Human articular chondrocytes are expanded to various extents as disclosed in example 1, analysed for the presence of markers associated with chondrocytic stability

according to example 4 and injected back in the cartilage lesion under the periosteal flap as described by Brittberg et al. (1996) *Clin. Orthop.*(326):270-83. After one month the animals are sacrificed and the joint surface defect analysed and scored by histology for the extent and quality of cartilage repair and for integration of the margins. *In situ* hybridisation for human *Alu* repeats is carried out in order to investigate the contribution of injected chondrocytes to the cartilage repair.

In a different approach we have devised an *ex vivo* model of JSD repair by ACT. The whole patella was excised from a male young New Zealand white rabbit, a cartilage defect was generated and previously isolated chondrocytes were injected underneath a periosteal flap sutured to cover the lesion. The patella was then placed in culture in DMEM supplemented with 10% FBS and antibiotic-antimycotic solution at 37°C in 5% CO₂ atmosphere. After 2 weeks the patella was fixed in 4% formaldehyde, imbedded in paraffin and analysed for histology and other techniques.

This setting allows tighter and more controlled experimental conditions and also a closer and much more flexible monitoring of the healing process by means of e.g. cell labelling, time point biopsy of the healing tissue for histologic and molecular analysis etc.

Example 6 - rescue of serially passaged articular chondrocytes

A short treatment with a growth factor from the TGF- β superfamily just before implantation can partially rescue serially passaged articular chondrocytes that have just lost phenotypic stability or reduce dramatically the cell expansion procedure occurring before cells can be injected for joint surface defects repair, ideally eliminating the need of it. The treatment is administered to cells in suspension for a short time and is followed by extensive washes in PBS just before injection. Similarly treated cells are tested for the expression of molecular markers linked to phenotypic stability of the articular chondrocyte.

In a set of experiments, freshly isolated articular chondrocytes in single cell suspension were exposed for 30 minutes to 100 ng/ml of CDMP-1 in Nutrient Mixture Ham's F-12 (Life Technologies) at 37°C, washed twice in PBS and injected in the *in vivo* assay of example 2. Control injections were made with chondrocytes exposed to HAM-F12 alone. After 3 weeks cartilage implants were

weighed, digested in 0.2% crude collagenase at 37°C and isolated cells were counted. The implant obtained from CDMP-1 treated cells had a wet weight three times higher as compared to samples treated with HAM-F12 alone, and cell count was twice as high. As shown in figure 6, also the production of highly sulphated proteoglycans was enhanced as witnessed by a more intense metachromatic staining with Safranin O in the implant obtained from CDMP-1 treated chondrocytes (Fig. 6B) as compared to control (Fig. 6A). This shows that a short exposure to CDMP-1 in suspension, just before injection, is capable of enhancing the chondrocytic phenotype as measured by the *in vivo* assay of example 2. Furthermore, the effectiveness of such short pulse makes prolonged, expensive and potentially dangerous expansions unnecessary.

Example 7 - Isolation of stable chondrocytes from a mixed cell population by the use of flow-cytometry

During cell expansion, as demonstrated in example 4, some chondrocytes become phenotypically unstable and unable to organise cartilage tissue *in vivo*. As a consequence, the chondrogenic potential of an expanded chondrocyte population depends not only from the mere number of cells but also from the number of phenotypically stable chondrocytes that it contains. The identification of membrane-associated molecular markers for both stable and unstable chondrocytes - for instance FGFR-3 and ALK-1 respectively - gives the opportunity to select optimal cells for ACT. The entire expanded cell population is incubated with antibodies directed to ALK-1 and FGFR-3 labelled with different fluorochromes. FACS analysis on double-labelled cells depicts the distribution of stable and unstable chondrocytes within the total pool. If needed, cell sorting is used to separate the stable from the unstable chondrocytes. A small aliquot of the sorted stable chondrocyte population is used for quality control using other independent positive and negative markers of chondrocyte stability (e.g. type II collagen and BMP-2 as positive markers and collagen type X as negative marker). The remaining stable chondrocytes are recovered in culture medium containing autologous serum and prepared for ACT. This allows obtaining a cell suspension composed of a consistent number of stable chondrocytes all contributing to cartilage repair and not a mixture of heterogeneous cells

27

regardless of their phenotype. It also allows eliminating from the pool unstable chondrocytes that not only are unable to generate cartilage *in vivo* but can potentially hamper the appropriate repair.

CLAIMS

1. An *in vivo* assay to measure anchorage-independent growth and phenotypic stability of a certain cell population comprising subcutaneous or
5 intramuscular injection in a mammal of a cell suspension of articular chondrocytes in an iso-osmotic liquid, the same suspension comprising articular chondrocytes in an amount equivalent to at least 1×10^6 chondrocytes as applied to immune-deficient mice.
2. Use of the *in vivo* assay of claim 1 to evaluate the possibility that a
10 treatment administered to a certain cell population can hamper or enhance the anchorage-independent growth of said population as well as its phenotypic stability.
3. Use of the *in vivo* assay of claim 1 to predict the outcome of autologous cell transplantation using a certain population of cells placed in a
15 certain differentiation pathway as a means to predict phenotypic stability.
4. Use of the *in vivo* assay of claim 1 to identify molecular markers linked to the phenotypic stability of a certain cell population in a certain differentiation pathway.
5. A method of identification of molecular markers linked to the
20 outcome of the *in vivo* assay of claim 1, comprising using freshly isolated or serially passaged cells from a certain cell population placed in a certain differentiation pathway.
6. Use of BMP-2 and/or FGFR-3 as molecular markers positively associated with chondrocyte phenotypic stability.
- 25 7. Use of activin-like kinase-1 (ALK-1) and/or type X collagen as molecular markers negatively associated with chondrocyte phenotypic stability.
8. Use of molecular markers of phenotype stability identified in claim 4 or claim 5 as tools to monitor passage by passage cell expansion and/or to predict when cell expansion must be stopped and/or to recover cells that have
30 already lost their phenotypic stability only when needed and/or to provide a means for quality control of cells to be used for autologous cell transplantation.
9. Use of molecular markers positively associated with phenotypic stability together with molecular markers negatively associated with phenotypic

stability as identified in claim 4 or claim 5 for selecting from a cell population only those cells that retain their phenotypic stability.

10. Use of cells retaining their phenotypic stability and selected according to claim 9 for transplantation to a connective tissue site in a patient or
5 for seeding any prosthetic device intended to be anchored into a mammal host.

11. A therapeutical composition including cells selected according to claim 9.

12. A therapeutical composition according to claim 11, further including at least a pharmaceutically acceptable carrier and/or a growth factor.

1

ABSTRACT

An *in vivo* assay to measure anchorage-independent growth and phenotypic stability of a certain cell population comprising subcutaneous or
5 intramuscular injection in a mammal of a cell suspension of articular chondrocytes in an iso-osmotic liquid, the same suspension comprising articular chondrocytes in an amount equivalent to at least 1×10^6 chondrocytes as applied to immune-deficient mice.

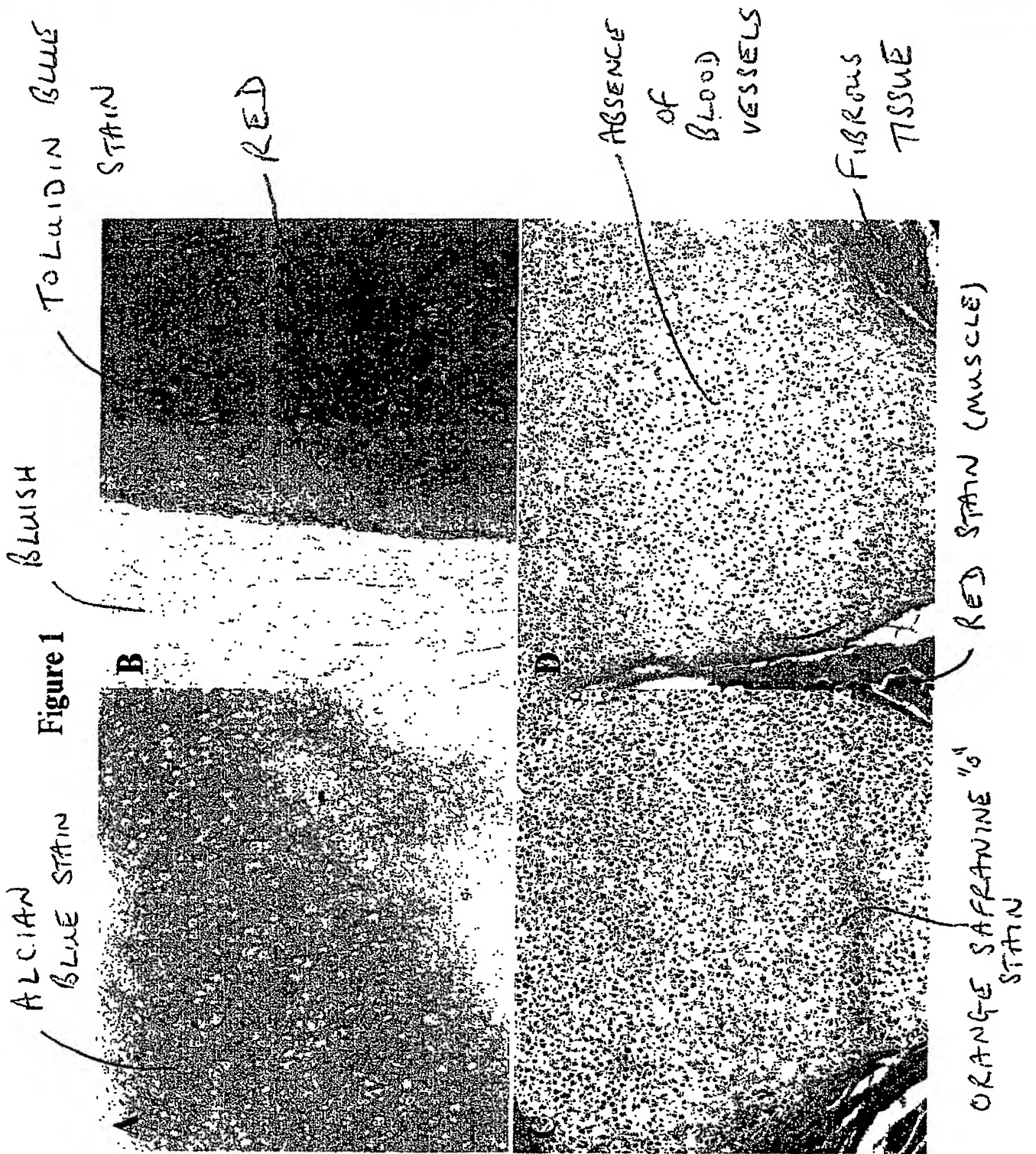


Figure 2

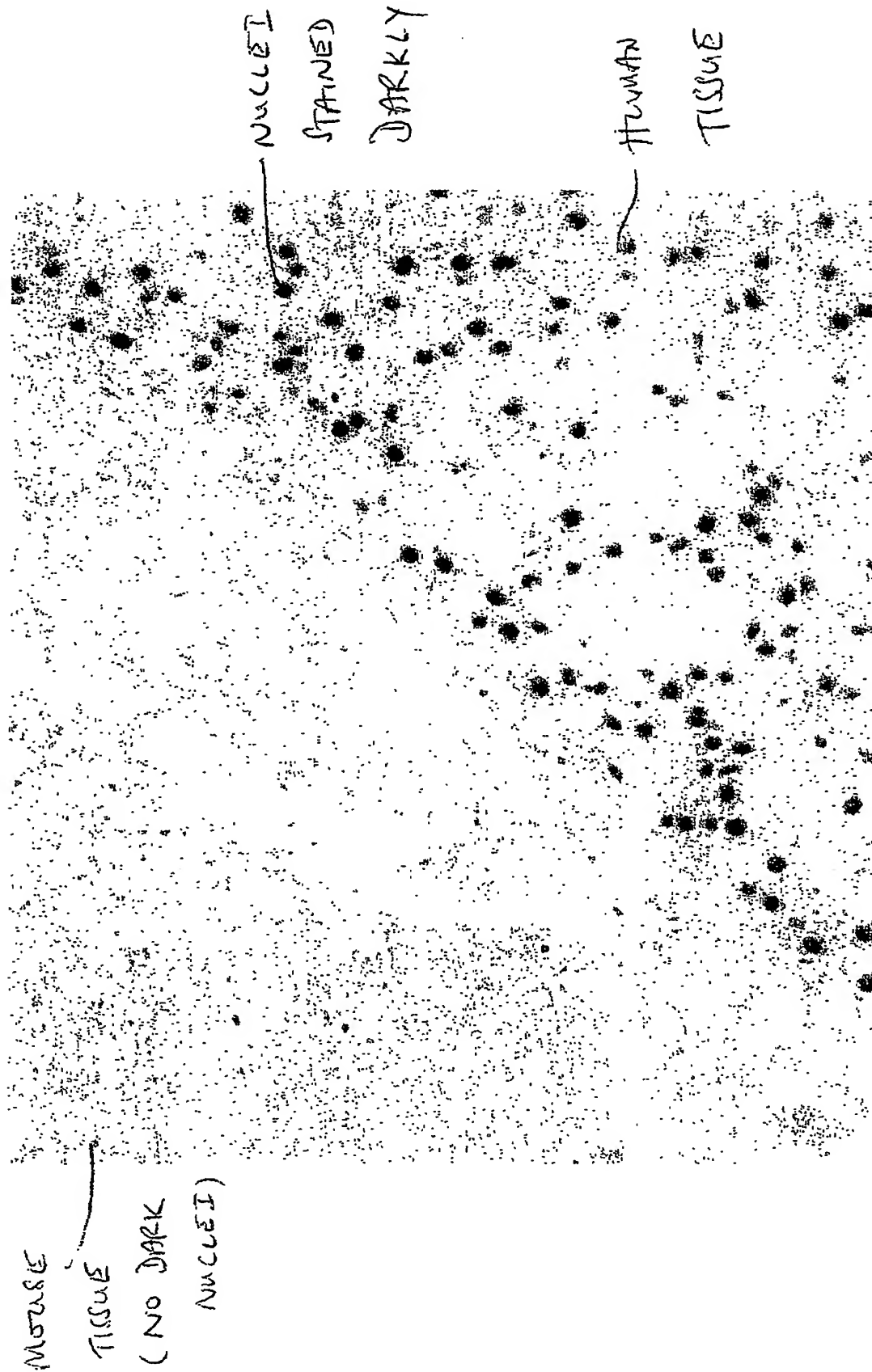
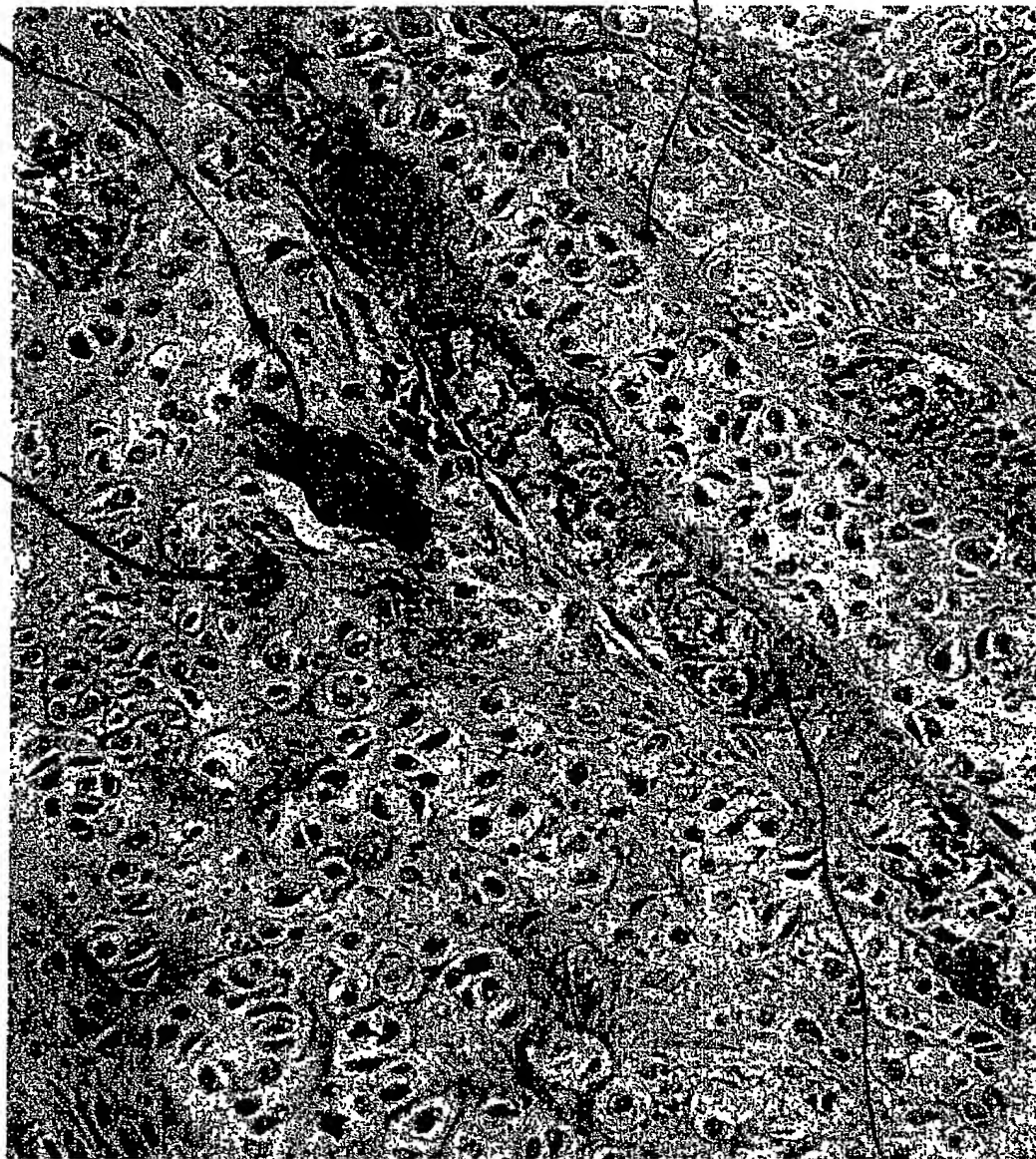


Figure 3

BRIGHT RED STAIN
(BLOOD VESSEL)
RED/BROWN
STAIN
(BONE)



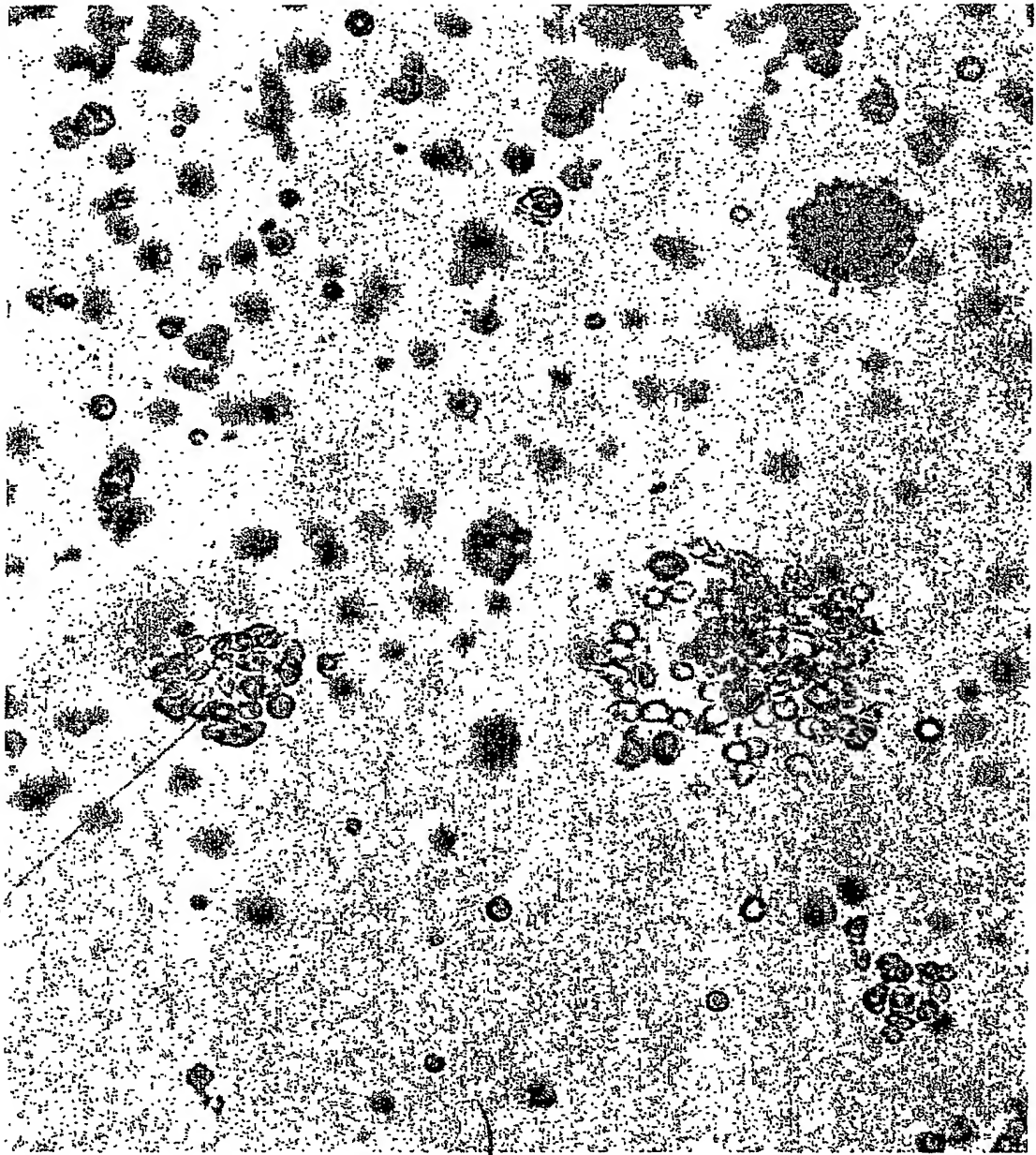
CARTILAGE

DARK
GREEN
STAIN
(BONE)

DARK
GREEN
STAIN
(BONE)

Figure 4

CHONDROCYTE COLONY



AGAR
MEDIUM

Figure 5

Collagen type II



BMP2



FGFR-3



Cdm p 1



Alk 1



Col lagen typeX



Beta actin



Figure 6

